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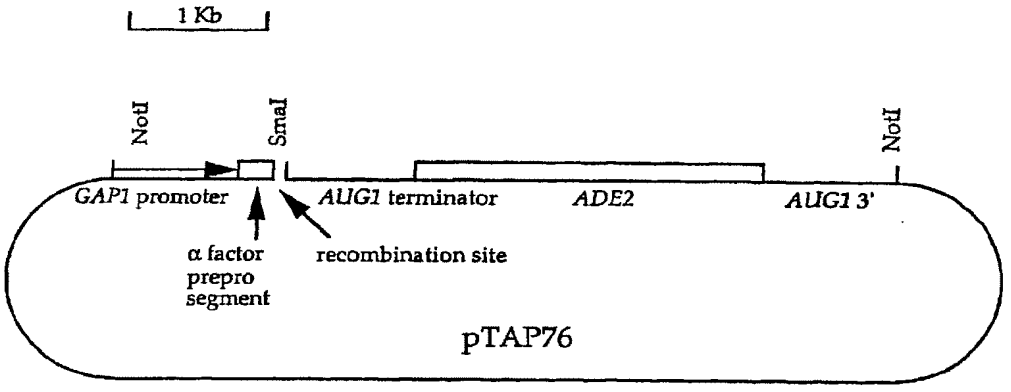

(57) Abstract: Transcription promoter and terminator sequences from the *Pichia methanolica* glyceraldehyde-3-phosphate dehydrogenase 1 gene (*GAP1 gene*) are disclosed. The sequences are useful within DNA constructs for the production of proteins of interest in cultured. *P. methanolica* cells. Within the expression vectors, a *GAP1* promoter and/or a *GAP1* terminator is operably linked to A DNA segment encoding the protein of interest.

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Description

PICHIA METHANOLICA GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE 1 PROMOTER AND TERMINATOR

BACKGROUND OF THE INVENTION

Methylotrophic yeasts are those yeasts that are able to utilize methanol as a sole source of carbon and energy. Species of yeasts that have the biochemical pathways necessary for methanol utilization are classified in four genera, Hansenula, Pichia, Candida, and Torulopsis. These genera are somewhat artificial, having been based on cell morphology and growth characteristics, and do not reflect close genetic relationships (Billon-Grand, Mycotaxon 35:201-204, 1989; Kurtzman, Mycologia 84:72-76, 1992). Furthermore, not all species within these genera are capable of utilizing methanol as a source of carbon and energy. As a consequence of this classification, there are great differences in physiology and metabolism between individual species of a genus.

Methylotrophic yeasts are attractive candidates for use in recombinant protein production systems for several reasons. First, some methylotrophic yeasts have been shown to grow rapidly to high biomass on minimal defined media. Second, recombinant expression cassettes are genomically integrated and therefore mitotically stable. Third, these yeasts are capable of secreting large amounts of recombinant proteins. See, for example, Faber et al., Yeast 11:1331, 1995; Romanos et al., Yeast 8:423, 1992; Cregg et al., Bio/Technology 11:905, 1993; U.S. Patent No. 4,855,242; U.S. Patent No. 4,857,467; U.S. Patent No. 4,879,231; and U.S. Patent No. 4,929,555; and Raymond, U.S. Patents Nos. 5,716,808, 5,736,383, 5,854,039, and 5,888,768.

Previously described expression systems for methylotrophic yeasts rely largely on the use of methanol-inducible transcription promoters. The use of methanol-induced promoters is, however, problematic as production is scaled up to commercial levels. The overall volume of methanol used during the fermentation process can be as much as 40% of the final fermentation volume, and at 1000-liter fermentation scale and above the volumes of methanol required for induction necessitate complex and potentially expensive considerations.

There remains a need in the art for additional materials and methods to enable the use of methylotrophic yeasts for production of polypeptides of economic

importance, including industrial enzymes and pharmaceutical proteins. The present invention provides such materials and methods as well as other, related advantages.

SUMMARY OF THE INVENTION

Within one aspect, the present invention provides an isolated DNA molecule of up to 1500 nucleotides in length comprising nucleotide 810 to nucleotide 1724 of SEQ ID NO:1.

Within a second aspect of the invention there is provided a DNA construct comprising the following operably linked elements: a first DNA segment comprising at least a portion of the sequence of SEQ ID NO:1 from nucleotide 733 to nucleotide 1732, wherein the portion is a functional transcription promoter; a second DNA segment encoding a protein of interest other than a *Pichia methanolica* glyceraldehyde-3-phosphate dehydrogenase; and a third DNA segment comprising a transcription terminator. Within one embodiment, the first DNA segment is from 900 to 1500 nucleotides in length. Within another embodiment, the first DNA segment is from 900 to 1000 nucleotides in length. Within a further embodiment, the first DNA segment comprises nucleotide 810 to nucleotide 1724 of SEQ ID NO:1. Within an additional embodiment, the first DNA segment is essentially free of DNA encoding a *P. methanolica* glyceraldehyde-3-phosphate dehydrogenase. The DNA construct may further comprise a selectable marker, such as a *P. methanolica* gene, for example a *P. methanolica ADE2* gene. The DNA construct may be a closed, circular molecule or a linear molecule. Within other embodiments, the DNA constuct further comprises a secretory signal sequence, such as a *Saccharomyces cerevisiae* alpha-factor pre-pro sequence, operably linked to the first and second DNA segments. Within additional embodiments, the third DNA segment comprises a transcription terminator of a *P. methanolica AUG1* or *GAP1* gene.

Within a third aspect of the invention there is provided a *P. methanolica* cell containing a DNA construct as disclosed above. Within one embodiment, the DNA construct is genomically integrated. Within a related embodiment, the DNA construct is genomically integrated in multiple copies. Within a further embodiment, the *P. methanolica* cell is functionally deficient in vacuolar proteases proteinase A and proteinase B.

Within a fourth aspect of the invention there is provided a method of producing a protein of interest comprising the steps of (a) culturing a *P. methanolica* cell as disclosed above whereby the second DNA segment is expressed and the protein of interest is produced, and (b) recovering the protein of interest.

Within a fifth aspect of the invention there is provided a DNA construct comprising the following operably linked elements: a first DNA segment comprising a *P. methanolica* gene transcription promoter; a second DNA segment encoding a protein of interest other than a *P. methanolica* protein; and a third DNA segment comprising nucleotides 2735 to 2795 of SEQ ID NO:1.

These and other aspects of the invention will become evident upon reference to the following detailed description of the invention and the attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the vector pBM/GAP, comprising the *P. methanolica GAP1* promoter.

Fig. 2 illustrates the vector pTAP76.

DETAILED DESCRIPTION OF THE INVENTION

The term "allelic variant" is used herein to denote an alternative form of a gene. Allelic variation is known to exist in populations and arises through mutation.

A "DNA construct" is a DNA molecule, either single- or double-stranded, that has been modified through human intervention to contain segments of DNA combined and juxtaposed in an arrangement not existing in nature.

A "DNA segment" is a portion of a larger DNA molecule having specified attributes. For example, a DNA segment encoding a specified polypeptide is a portion of a longer DNA molecule, such as a plasmid or plasmid fragment, that, when read from the 5' to the 3' direction, encodes the sequence of amino acids of the specified polypeptide.

The term "functionally deficient" denotes the expression in a cell of less than 10% of an activity as compared to the level of that activity in a wild-type counterpart. Often the expression level will be less than 1% of the activity in the wild-type counterpart, frequently less than 0.01% as determined by appropriate assays. In some instances it is desirable that the activity be essentially undetectable (i.e., not significantly above background). Functional deficiencies in genes can be generated by mutations in either coding or non-coding regions.

The term "gene" is used herein to denote a DNA segment encoding a polypeptide. Where the context allows, the term includes genomic DNA (with or without intervening sequences), cDNA, and synthetic DNA. Genes may include non-coding sequences, including promoter elements.

The term "isolated", when applied to a polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones.

"Operably linked", when referring to DNA segments, indicates that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator.

A "polynucleotide" is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When these terms are applied to double-stranded molecules they are used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

The term "promoter" is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes. Sequences within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. These promoter elements include RNA polymerase binding sites, TATA sequences, and transcription factor binding sites. Of particular interest within the present invention are Gcr1p binding sites, characterized by the consensus sequences CTTCC or GGAAG, and Rap1p binding sites. See, in general, Watson et al., eds., Molecular Biology of the Gene, 4th ed., The Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA, 1987.

A "pro sequence" is a DNA sequence that commonly occurs immediately 5' to the mature coding sequence of a gene encoding a secretory protein. The pro sequence encodes a pro peptide that serves as a cis-acting chaperone as the protein moves through the secretory pathway.

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are commonly defined in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway. A secretory peptide and a pro peptide may be collectively referred to as a pre-pro peptide.

The present invention provides isolated DNA molecules comprising a *Pichia methanolica* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene promoter. The invention also provides isolated DNA molecules comprising a *P. methanolica* GAPDH gene terminator. The promoter and terminator can be used within methods of producing proteins of interest, including proteins of pharmaceutical or industrial value.

The sequence of a DNA molecule comprising a *P. methanolica* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene promoter, coding region, and terminator is shown in SEQ ID NO:1. The gene has been designated *GAP1*. those skilled in the art will recognize that SEQ ID NO:1 represents a single allele of the *P. methanolica GAP1* gene and that other functional alleles (allelic variants) are likely to exist, and that allelic variation may include nucleotide changes in the promoter region, coding region, or terminator region.

Within SEQ ID NO:1, the GAPDH open reading frame begins with the methionine codon (ATG) at nucleotides 1733 - 1735. The transcription promoter is located upstream of the ATG. Gene expression experiments showed that a functional promoter was contained within the ca. 900 nucleotide 5'-flanking region of the *GAP1* gene. Analysis of this promoter sequence revealed the presence of a number of sequences homologous to *Saccharomyces cerevisiae* promoter elements. These sequences include a concensus TATAAA box at nucleotides 1584 to 1591, a consensus

Rap1p binding site (Graham and Chambers, *Nuc. Acids Res.* 22:124-130, 1994) at nucleotides 1355 to 1367, and potential Gcr1p binding sites (Shore, *Trends Genet.* 10:408-412, 1994) at nucleotides 1225 to 1229, 1286 to 1290, 1295 to 1299, 1313 to 1317, 1351 to 1354, 1370 to 1374, 1389 to 1393, and 1457 to 1461. While not wishing to be bound by theory, it is believed that these sequences may perform functions similar to those of their counterparts in the *S. cerevisiae TDH3* promoter (Bitter et al., *Mol. Gen. Genet.* 231:22-32, 1991), that is, they may bind the homologous transcription regulatory elements. Mutation of the region around the consensus Gcr1p binding site in the *P. methanolica GAP1* promoter has been found to destroy promoter activity.

Preferred portions of the sequence shown in SEQ ID NO:1 for use within the present invention as transcription promoters include segments comprising at least 900 contiguous nucleotides of the 5' non-coding region of SEQ ID NO:1, and preferably comprising nucleotide 810 to nucleotide 1724 of the sequence shown in SEQ ID NO:1. Those skilled in the art will recognize that longer portions of the 5' non-coding region of the *P. methanolica GAP1* gene can also be used. Promoter sequences of the present invention can thus include the sequence of SEQ ID NO:1 through nucleotide 1732 in the 3' direction and can extend to or beyond nucleotide 232 in the 5' direction. For convenience and ease of manipulation, the promoter used within an expression DNA construct will generally not exceed 1.5 kb in length, and will often not exceed 1.0 kb in length.

As disclosed in more detail in the examples that follow, the sequence of SEQ ID NO:1 from nucleotide 810 to 1724 provides a functional transcription promoter. However, additional nucleotides can be removed from either or both ends of this sequence and the resulting sequence tested for promoter function by joining it to a sequence encoding a protein, preferably a protein for which a convenient assay is readily available.

Within the present invention it is preferred that the *GAP1* promoter be substantially free of *GAP1* gene coding sequence, which begins with nucleotide 1733 in SEQ ID NO:1. As used herein, the term "substantially free of *GAP1* gene coding sequence" means that the promoter DNA includes not more than 15 nucleotides of the *GAP1* coding sequences, preferably not more than 10 nucleotides, and more preferably not more than 3 nucleotides. Within one embodiment of the invention, the *GAP1* promoter is provided free of coding sequence of the *P. methanolica GAP1* gene. However, those skilled in the art will recognize that a *GAP1* gene fragment that includes the initiation ATG (nucleotides 1733 to 1735) of SEQ ID NO:1 can be operably linked to a heterologous coding sequence that lacks an ATG, with the *GAP1* ATG providing for initiation of translation of the heterologous sequence. Those skilled

in the art will further recognize that additional *GAP1* coding sequences can also be included, whereby a fusion protein comprising *GAP1* and heterologous amino acid sequences is produced. Such a fusion protein may comprise a cleavage site to facilitate separation of the *GAP1* and heterologous sequences subsequent to translation.

In addition to the *GAP1* promoter sequence, the present invention also provides transcription terminator sequences derived from the 3' non-coding region of the *P. methanolica GAP1* gene. A consensus transcription termination sequence (Chen and Moore, *Mol. Cell. Biol.* 12:3470-3481, 1992) is at nucleotides 2774 to 2787 of SEQ ID NO:1. Within the present invention, there are thus provided transcription terminator gene segments of at least about 60 bp in length. Longer segments, for example at least 90 bp in length or about 200 bp in length, will often be used. These segments comprise the termination sequence disclosed above, and may have as their 5' termini nucleotide 2735 of SEQ ID NO:1. Those skilled in the art will recognize, however, that the transcription terminator segment that is provided in an expression vector can include at its 5' terminus the TAA translation termination codon at nucleotides 2732-2734 of SEQ ID NO:1 to permit the insertion of coding sequences that lack a termination codon.

Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are well known in the art and are disclosed by, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; Murray, ed., Gene Transfer and Expression Protocols, Humana Press, Clifton, NJ, 1991; Glick and Pasternak, Molecular Biotechnology: Principles and Applications of Recombinant DNA, ASM Press, Washington, D.C., 1994; Ausubel et al. (eds.), Short Protocols in Molecular Biology, 3rd edition, John Wiley and Sons, Inc., NY, 1995; Wu et al., Methods in Gene Biotechnology, CRC Press, New York, 1997. DNA vectors, including expression vectors, commonly contain a selectable marker and origin of replication that function in a bacterial host (e.g., *E. coli*) to permit the replication and amplification of the vector in a prokaryotic host. If desired, these prokaryotic elements can be removed from a vector before it is introduced into an alternative host. For example, such prokaryotic sequences can be removed by linearization of the vector prior to its introduction into a *P. methanolica* host cell.

Within one embodiment of the invention, expression vectors are provided that comprise a first DNA segment comprising at least a portion of the sequence of SEQ ID NO:1 that is a functional transcription promoter operably linked to a second DNA segment encoding a protein of interest. When it is desired to secrete the protein of interest, the vector will further comprise a secretory signal sequence operably

linked to the first and second DNA segments. The secretory signal sequence may be that of the protein of interest, or may be derived from another secreted protein, preferably a secreted yeast protein. A preferred such yeast secretory signal sequence is the *S. cerevisiae* alpha-factor (*MF*α1) pre-pro sequence (disclosed by Kurjan et al., U.S. Patent No. 4,546,082 and Brake, U.S. Patent No. 4,870,008).

Within other embodiments of the invention, expression vectors are provided that comprise a DNA segment comprising a portion of SEQ ID NO:1 that is a functional transcription terminator operably linked to an additional DNA segment encoding a protein of interest. Within one embodiment, the *P. methanolica GAP1* promoter and terminator sequences are used in combination, wherein both are operably linked to a DNA segment encoding a protein of interest within an expression vector.

Expression vectors of the present invention further comprise a selectable marker to permit identification and selection of *P. methanolica* cells containing the vector. Selectable markers provide for a growth advantage of cells containing them. The general principles of selection are well known in the art. The selectable marker is preferably a *P. methanolica* gene. Commonly used selectable markers are genes that encode enzymes required for the synthesis of amino acids or nucleotides. Cells having mutations in these genes cannot grow in media lacking the specific amino acid or nucleotide unless the mutation is complemented by the selectable marker. Use of such "selective" culture media ensures the stable maintenance of the heterologous DNA within the host cell. An exemplary selectable marker of this type for use in *P. methanolica* is a *P. methanolica ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21). See, Raymond, U.S. Patent No. 5,736,383. The *ADE2* gene, when transformed into an *ade2* host cell, allows the cell to grow in the absence of adenine. The coding strand of a representative *P. methanolica ADE2* gene sequence is shown in SEQ ID NO:2. The sequence illustrated includes 1006 nucleotides of 5' non-coding sequence and 442 nucleotides of 3' non-coding sequence, with the initiation ATG codon at nucleotides 1007-1009. Within one embodiment of the invention, a DNA segment comprising nucleotides 407-2851 is used as a selectable marker, although longer or shorter segments could be used as long as the coding portion is operably linked to promoter and terminator sequences. In the alternative, a dominant selectable marker, which provides a growth advantage to wild-type cells, may be used. Typical dominant selectable markers are genes that provide resistance to antibiotics, such as neomycin-type antibiotics (e.g., G418), hygromycin B, and bleomycin/phleomycin-type antibiotics (e.g., Zeocin™; available from Invitrogen Corporation, San Diego, CA). An exemplary dominant selectable marker for use in *P. methanolica* is the *Sh bla* gene, which inhibits the activity of Zeocin™.

The use of *P. methanolica* cells as a host for the production of recombinant proteins is disclosed in WIPO Publications WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565; and U.S. Patents Nos. 5,716,808, 5,736,383, 5,854,039, and 5,888,768. Expression vectors for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. To facilitate integration of the expression vector DNA into the host chromosome, the entire expression segment of the plasmid can be flanked at both ends by host DNA sequences (e.g., *AUG1* 3' sequences). Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. It is preferred to transform *P. methanolica* cells by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (τ) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

Integrative transformants are preferred for use in protein production processes. Such cells can be propagated without continuous selective pressure because DNA is rarely lost from the genome. Integration of DNA into the host chromosome can be confirmed by Southern blot analysis. Briefly, transformed and untransformed host DNA is digested with restriction endonucleases, separated by electrophoresis, blotted to a support membrane, and probed with appropriate host DNA segments. Differences in the patterns of fragments seen in untransformed and transformed cells are indicative of integrative transformation. Restriction enzymes and probes can be selected to identify transforming DNA segments (e.g., promoter, terminator, heterologous DNA, and selectable marker sequences) from among the genomic fragments.

Differences in expression levels of heterologous proteins can result from such factors as the site of integration and copy number of the expression cassette among individual isolates. It is therefore advantageous to screen a number of isolates for expression level prior to selecting a production strain. Isolates exhibiting a high expression level will commonly contain multiple integrated copies of the desired expression cassette. A variety of suitable screening methods are available. For example, transformant colonies are grown on plates that are overlayed with membranes (e.g., nitrocellulose) that bind protein. Proteins are released from the cells by secretion or following lysis, and bind to the membrane. Bound protein can then be assayed using known methods, including immunoassays. More accurate analysis of expression levels can be obtained by culturing cells in liquid media and analyzing conditioned media or cell lysates, as appropriate. Methods for concentrating and purifying proteins from

media and lysates will be determined in part by the protein of interest. Such methods are readily selected and practiced by the skilled practitioner.

For production of secreted proteins, host cells having functional deficiencies in the vacuolar proteases proteinase A, which is encoded by the *PEP4* gene, and proteinase B, which is encoded by the *PRB1* gene, can be used to minimize spurious proteolysis. Vacuolar protease activity (and therefore vacuolar protease deficiency) is measured using any of several known assays, such as those developed for *S. cerevisiae* and disclosed by Jones, *Methods Enzymol.* 194:428-453, 1991. One such assay is the APNE overlay assay, which detects activity of carboxypeptidase Y (CpY). See, Wolf and Fink, *J. Bact.* 123:1150-1156, 1975. Because the zymogen (pro)CpY is activated by proteinase A and proteinase B, the APNE assay is indicative of vacuolar protease activity in general. The APNE overlay assay detects the carboxypeptidase Y-mediated release of β-naphthol from N-acetyl-phenylalanine-β-naphthyl-ester (APNE), which results in the formation of an isoluble red dye by the reaction of the β-naphthol with the diazonium salt Fast Garnet GBC. Cells growing on assay plates (e.g., YEPD plates) at room temperature are overlayed with 8 ml RxM. RxM is prepared by combining 0.175 g agar, 17.5 ml H_2O, and 5 ml 1 M Tris-HCl pH 7.4, microwaving the mixture to dissolve the agar, cooling to ~55°C, adding 2.5 ml freshly made APNE (2 mg/ml in dimethylformamide) (Sigma Chemical Co., St. Louis, MO), and, immediately before assay, 20 mg Fast Garnet GBC salt (Sigma Chemical Co.). The overlay is allowed to solidify, and color development is observed. Wild-type colonies are red, whereas CPY deletion strains are white. Carboxypeptidase Y activity can also be detected by the well test, in which cells are distributed into wells of a microtiter test plate and incubated in the presence of *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTPNA) and dimethylformamide. The cells are permeabilized by the dimethylformamide, and CpY in the cells cleaves the amide bond in the BTPNA to give the yellow product *p*-nitroaniline. Assays for CpY will detect any mutation that reduces protease activity so long as that activity ultimately results in the reduction of CpY activity.

P. methanolica cells are cultured in a medium comprising adequate sources of carbon, nitrogen and trace nutrients at a temperature of about 25°C to 35°C. Liquid cultures are provided with sufficient aeration by conventional means, such as shaking of small flasks or sparging of fermentors. A suitable culture medium for *P. methanolica* is YEPD (2% D-glucose, 2% Bacto™ Peptone (Difco Laboratories, Detroit, MI), 1% Bacto™ yeast extract (Difco Laboratories), 0.004% adenine, 0.006% L-leucine).

For large-scale culture, one to two colonies of a *P. methanolica* strain can be picked from a fresh agar plate (e.g, YEPD agar) and suspended in 250 ml of

YEPD broth contained in a two-liter baffled shake flask. The culture is grown for 16 to 24 hours at 30°C and 250 rpm shaking speed. Approximately 50 to 80 milliliters of inoculum are used per liter starting fermentor volume (5 - 8% v/v inoculum).

A preferred fermentation medium is a soluble medium comprising glucose as a carbon source, inorganic ammonia, potassium, phosphate, iron, and citric acid. As used herein, a "soluble medium" is a medium that does not contain visible precipitation. Preferably, the medium lacks phosphate glass (sodium hexametaphosphate). A preferred medium is prepared in deionized water and does not contain calcium sulfate. As a minimal medium, it is preferred that the medium lacks polypeptides or peptides, such as yeast extracts. However, acid hydrolyzed casein *(e.g.,* casamino acids or amicase) can be added to the medium if desired. An illustrative fermentation medium is prepared by mixing the following compounds: $(NH_4)_2SO_4$ (11.5 grams/liter), K_2HPO_4 (2.60 grams/liter), KH_2PO_4 (9.50 grams/liter), $FeSO_4 \cdot 7H_2O$ (0.40 grams/liter), and citric acid (1.00 gram/liter). After adding distilled, deionized water to one liter, the solution is sterilized by autoclaving, allowed to cool, and then supplemented with the following: 60% (w/v) glucose solution (47.5 milliliters/liter), 10x trace metals solution (20.0 milliliters/liter), 1 M $MgSO_4$ (20.0 milliliters/liter), and vitamin stock solution (2.00 milliliters/liter). The 10x trace metals solution contains $FeSO_4 \cdot 7H_2O$ (100 mM), $CuSO_4 \cdot 5H_2O$ (2 mM), $ZnSO_4 \cdot 7H_2O$ (8 mM), $MnSO_4 \cdot H_2O$ (8 mM), $CoCl_2 \cdot 6H_2O$ (2 mM), $Na_2MoO_4 \cdot 2H_2O$ (1 mM), H_3BO_3 (8 mM), KI (0.5 mM), $NiSO_4 \cdot 6H_2O$ (1 mM), thiamine (0.50 grams/liter), and biotin (5.00 milligrams/liter). The vitamin stock solution contains inositol (47.00 grams/liter), pantothenic acid (23.00 grams/liter), pyrodoxine (1.20 grams/liter), thiamine (5.00 grams/liter), and biotin (0.10 gram/liter). Those of skill in the art can vary these particular ingredients and amounts. For example, ammonium sulfate can be substituted with ammonium chloride, or the amount of ammonium sulfate can be varied, for example, from about 11 to about 22 grams/liter.

After addition of trace metals and vitamins, the pH of the medium is typically adjusted to pH 4.5 by addition of 10% H_3PO_4. Generally, about 10 milliliters/liter are added, and no additional acid addition will be required. During fermentation, the pH is maintained between about 3.5 to about 5.5, or about 4.0 to about 5.0, depending on protein produced, by addition of 5 N NH_4OH.

An illustrative fermentor is a BIOFLO 3000 fermentor system (New Brunswick Scientific Company, Inc.; Edison, NJ). This fermentor system can handle either a six-liter or a fourteen-liter fermentor vessel. Fermentations performed with the six-liter vessel are prepared with three liters of medium, whereas fermentations performed with the fourteen-liter vessel are prepared with six liters of medium. The

fermentor vessel operating temperature is typically set to 30°C for the course of the fermentation, although the temperature can range between 27-31°C depending on the protein expressed. The fermentation is initiated in a batch mode. The glucose initially present is often used by approximately 10 hours elapsed fermentation time (EFT), at which time a glucose feed can be initiated to increase the cell mass. An illustrative glucose feed contains 900 milliliters of 60% (w/v) glucose, 60 milliliters of 50% (w/v) $(NH_4)_2SO_4$, 60 milliliters of 10x trace metals solution, and 30 milliliters of 1 M $MgSO_4$. *P. methanolica* fermentation is robust and requires high agitation, aeration, and oxygen sparging to maintain the percentage dissolved oxygen saturation above 30%. The percentage dissolved oxygen should not drop below 15% for optimal expression and growth. The biomass typically reaches about 30 to about 80 grams dry cell weight per liter at 48 hours EFT.

Proteins produced according to the present invention are recovered from the host cells using conventional methods. If the protein is produced intracellulary, the cells are harvested (e.g., by centrifugation) and lysed to release the cytoplasmic contents. Methods of lysis include enzymatic and mechanical disruption. The crude extract is then fractionated according to known methods, the specifics of which will be determined for the particular protein of interest. Secreted proteins are recovered from the conditioned culture medium using standard methods, also selected for the particular protein. See, in general, Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York, 1994.

The materials and methods of the present invention can be used to produce proteins of research, industrial, or pharmaceutical interest. Such proteins include enzymes, such as lipases, cellulases, and proteases; enzyme inhibitors, including protease inhibitors; growth factors such as platelet derived growth factor (PDGF), fibroblast growth factors (FGF), epidermal growth factor (EGF), vascular endothelial growth factors (VEGFs); glutamic acid decarboxylase (GAD); cytokines, such as erythropoietin, thrombopoietin, colony stimulating factors, interleukins, and interleukin antagonists; hormones, such as insulin, proinsulin, leptin, and glucagon; and receptors, including growth factor receptors, which can be expressed in truncated form ("soluble receptors") or as fusion proteins with, for example, immunoglobulin constant region sequences. DNAs encoding these and other proteins are known in the art. See, for example, U.S. Patents Nos. 4,889,919; 5,219,759; 4,868,119; 4,968,607; 4,599,311; 4,784,950; 5,792,850; 5,827,734; 4,703,008; 4,431,740; and 4,762,791; and WIPO Publications WO 95/21920 and WO 96/22308.

The materials and methods of the present invention can be used to produce unglycosylated pharmaceutical proteins. Yeast cells, including *P. methanolica*

cells, produce glycoproteins with carbohydrate chains that differ from their mammalian counterparts. Mammalian glycoproteins produced in yeast cells may therefore be regarded as "foreign" when introduced into a mammal, and may exhibit, for example, different pharmacokinetics than their naturally glycosylated counterparts.

The invention is further illustrated by the following, non-limiting examples.

EXAMPLES

Example 1

To clone the *P. methanolica GAP1* gene, sense (ZC11,356; SEQ ID NO:3) and antisense (ZC11,357; SEQ ID NO:4) PCR primers were designed from an alignment of the coding regions of GAPDH genes of *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, and mouse. The primers were then used to amplify *P. methanolica* genomic DNA. An amplified sequence 608 bp long was recovered and was found to have 78.1% homology to the corresponding *S. cerevisiae* GAPDH gene sequence.

A *P. methanolica* genomic library was constructed in the vector pRS426 (Christianson et al., *Gene* 110:119-122, 1992), a shuttle vector comprising 2μ and *S. cerevisiae URA3* sequences, allowing it to be propagated in *S. cerevisiae*. Genomic DNA was prepared from strain CBS6515 according to standard procedures. Briefly, cells were cultured overnight in rich media, spheroplasted with zymolyase, and lysed with SDS. DNA was precipitated from the lysate with ethanol and extracted with a phenol/chloroform mixture, then precipitated with ammonium acetate and ethanol. Gel electrophoresis of the DNA preparation showed the presence of intact, high molecular weight DNA and appreciable quantities of RNA. The DNA was partially digested with Sau 3A by incubating the DNA in the presence of a dilution series of the enzyme. Samples of the digests were analyzed by electrophoresis to determine the size distribution of fragments. DNA migrating between 4 and 12 kb was cut from the gel and extracted from the gel slice. The size-fractionated DNA was then ligated to pRS426 that had been digested with Bam HI and treated with alkaline phosphatase. Aliquots of the reaction mixture were electroporated into *E. coli* MC1061 cells using an electroporator (Gene Pulser™; BioRad Laboratories, Hercules, CA) as recommended by the manufacturer.

The library was screened by PCR using sense (ZC11,733; SEQ ID NO:5) and antisense (ZC11,734; SEQ ID NO:6) primers designed from the sequenced region of the *P. methanolica* GAPDH gene fragment. The PCR reaction mixture was incubated for one minute at 94°C; followed by 34 cycles of 94°C, one minute, 52°C, 45

seconds, 72°C, two minutes; and a termination cycle of 94°C, one minute, 54°C, one minute, 72°C, eleven minutes. Starting with 43 library pools, positive pools were identified and broken down to individual colonies. A single colony with a pRS426 plasmid containing the *P. methanolica* GAPDH gene as its insert was isolated. The orientation of the GAPDH gene and the length of the 5' and 3' flanking sequences in the insert were deduced by DNA sequencing (SEQ ID NO:1). This gene was designated *GAP1*.

A plasmid containing the *GAP1* gene, designated pGAPDH, has been deposited as an *E. coli* strain MC1061 transformant with American Type Culture Collection, Manassas, VA under the terms of the Budapest Treaty. The deposited strain has been assigned the designation PTA-3 and a deposit date of May 4, 1999.

Example 2

The cloned *P. methanolica GAP1* promoter was used to construct an expression cassette by replacing the *AUG1* promoter in the vector pCZR133 (disclosed in U.S. Patent No. 5,736,383). Plasmid pCZR133 comprises the *P. methanolica AUG1* promoter and terminator flanking a multiple cloning site, and a *P. methanolica ADE2* selectable marker. The *GAP1* promoter (nucleotides 810 to 1724 of SEQ ID NO:1) was amplified by PCR using primers that introduced a Not I site at the 5' end (SEQ ID NO:7; ZC12,586), and Eco RI and Bam HI sites at the 3' end (SEQ ID NO:8; ZC12,565). The reaction mixture was incubated for one minute at 94°C; followed by 34 cycles of 94°C, one minute, 52°C, one minute, 72°C, three minutes; and a termination cycle of 94°C, one minute, 54°C, seven minutes, 72°C, 23 minutes. The amplified promoter was then blunt-end ligated into a phagemid vector (pBluescript®; Stratagene, La Jolla, CA). The orientation of the promoter in the vector was determined by restriction analysis. The promoter was isolated as a Not I - Bam HI fragment. Plasmid pCZR133 was digested with Not I and Bam HI, and the digest was electrophoresed on a gel. Two fragments, the Ade2/termination fragment and the pUC fragment, were recovered. The pUC fragment was dephosphorylated. The two vector fragments and the promoter were joined in a three-part ligation. The resulting plasmid was designated pBM/GAP (Fig. 1).

A second vector, pTAP76 (Fig. 2) was constructed. This vector comprises the *GAP1* promoter, α-factor prepro sequence, a SmaI cleavage site, the *AUG1* terminator, the *ADE2* selectable marker, and *AUG1* 3' non-coding sequence cloned into a pRS316 (Sikorski and Hieter, *Genetics* 122:19-27, 1989) backbone. The pTAP76 vector is linearized at the SmaI site and combined with a DNA fragment of interest and double-stranded recombination linkers in *S. cerevisiae*, whereby the

fragment of interest is joined to the vector by homologous recombination as disclosed by Raymond et al., *BioTechniques* 26:134-141, 1999.

Example 3

Expression of heterologous genes from the *GAP1* promoter was tested using LacZ and GFP (green fluorescent protein) reporter genes. These genes were prepared as Eco RI-Bam HI fragments, and were individually ligated to Eco RI, Bam HI-digested pBM/GAP. The resulting plasmids were transformed into *P. methanolica* host cells, and the cells were grown in both glucose and methanol fermentation conditions. Both reporter genes were expressed under both conditions, showing that the cloned *GAP1* promoter can be used to constitutively express heterologous genes in *P. methanolica* cells.

Example 4

To generate a *P. methanolica* strain deficient for vacuolar proteases, the *PEP4* and *PRB1* genes were identified and disrupted. *PEP4* and *PRB1* sequences were amplified by PCR in reaction mixtures containing 100 pmol of primer DNA, 1X buffer as supplied (Boehringer Mannheim, Indianapolis, IN), 250 μM dNTPs, 1-100 pmol of template DNA, and 1 unit of Taq polymerase in a reaction volume of 100 μl. The DNA was amplified over 30 cycles of 94°C, 30 seconds; 50°C, 60 seconds; and 72°C, 60 seconds.

Using an alignment of *PEP4* sequences derived from *S. cerevisiae* (Ammerer et al., *Mol. Cell. Biol.* 6:2490-2499, 1986; Woolford et al., *Mol. Cell. Biol.* 6:2500-2510, 1986) and *P. pastoris* (Gleeson et al., U.S. Patent No. 5,324,660), several sense and antisense primers corresponding to conserved regions were designed. One primer set, ZC9118 (SEQ ID NO:9) and ZC9464 (SEQ ID NO:10) produced a PCR product of the expected size from genomic DNA, and this set was used to identify a genomic clone corresponding to the amplified region. DNA sequencing of a portion of this genomic clone (shown in SEQ ID NO:11) revealed an open reading frame encoding a polypeptide (SEQ ID NO:12) with 70% amino acid identity with proteinase A from *S. cerevisiae*.

Primers for the identification of *P. methanolica PRB1* were designed on the basis of alignments between the *PRB1* genes of *S. cerevisiae* (Moehle et al., *Mol. Cell. Biol.* 7:4390-4399, 1987), *P. pastoris* (Gleeson et al., U.S. Pat. No. 5,324,660), and *Kluyveromyces lactis* (Fleer et al., WIPO Publication WO 94/00579). One primer set, ZC9126 (SEQ ID NO:13) and ZC9741 (SEQ ID NO:14) amplified a ca. 400 bp fragment from genomic DNA (SEQ ID NO:15). This product was sequenced and found

to encode a polypeptide (SEQ ID NO:16) with 70% amino acid identity with proteinase B from *S. cerevisiae*. The PRB primer set was then used to identify a genomic clone encompassing the *P. methanolica PRB1* gene.

Deletion mutations in the *P. methanolica PEP4* and *PRB1* genes were generated using available restriction enzyme sites. The cloned genes were restriction mapped. The *pep4Δ* allele was created by deleting a region of approximately 500 bp between BamHI and NcoI sites and including nucleotides 1 through 393 the sequence shown in SEQ ID NO:11. The *prb1Δ* allele was generated by deleting a region of approximately 1 kbp between NcoI and EcoRV sites and including the sequence shown in SEQ ID NO:15. The cloned *PEP4* and *PRB1* genes were subcloned into pCZR139, a phagemid vector (pBluescript® II KS(+), Stratagene, La Jolla, CA) that carried a 2.4 kb SpeI *ADE2* insert, to create the deletions. In the case of *PEP4* gene, the unique BamHI site in pCZR139 was eliminated by digestion, fill-in, and religation. The vector was then linearized by digestion with EcoRI and HindIII, and a ca. 4 kb EcoRI - HindIII fragment spanning the *PEP4* gene was ligated to the linearized vector to produce plasmid pCZR142. A ca. 500-bp deletion was then produced by digesting pCZR142 with BamHI and NcoI, filling in the ends, and religating the DNA to produce plasmid pCZR143. The *PRB1* gene (~5 kb XhoI - BamHI fragment) was subcloned into pCZR139, and an internal EcoRV - NcoI fragment, comprising the sequence shown in SEQ ID NO:15, was deleted to produce plasmid pCZR153.

Plasmid pCZR143 was linearized with Asp718, which cut at a unique site. The linearized plasmid was introduced into the *P. methanolica* PMAD11 strain (an *ade2* mutant generated as disclosed in U.S. Patent No. 5,736,383). Transformants were grown on ADE DS (Table 1) to identify Ade^+ transformants. Two classes of white, Ade^+ transformants were analyzed. One class arose immediately on the primary transformation plate; the scond became evident as rapidly growing white papillae on the edges of unstable, pink transformant colonies.

Table 1

ADE DS

0.056% -Ade -Trp -Thr powder
0.67% yeast nitrogen base without amino acids
2% D-glucose
0.5% 200X tryptophan, threonine solution
18.22% D-sorbitol

-Ade -Trp -Thr powder

powder made by combining 3.0 g arginine, 5.0 g aspartic acid, 2.0 g histidine, 6.0 g isoleucine, 8.0 g leucine, 4.0 g lysine, 2.0 g methionine, 6.0 g phenylalanine, 5.0 g serine, 5.0 g tyrosine, 4.0 g uracil, and 6.0 g valine (all L-amino acids)

200X tryptophan, threonine solution

3.0% L-threonine, 0.8% L-tryptophan in H_2O

For plates, add 1.8% Bacto™ agar (Difco Laboratories)

Southern blotting was used to identify transformants that had undergone the desired homologous integration event. 100 µl of cell paste was scraped from a 24-48 hour YEPD plate and washed in 1 ml water. Washed cells were resuspended in 400 µl of spheroplast buffer (1.2 M sorbitol, 10 mM Na citrate pH 7.5, 10 mM EDTA, 10 mM DTT, 1 mg/ml zymolyase 100T) and incubated at 37°C for 10 minutes. Four hundred µl of 1% SDS was added, the cell suspension was mixed at room temperature until clear, 300 µl of 5 M potassium acetate was mixed in, and the mixture was clarified by microcentrifugation for 5 minutes. 750 µl of the clarified lysate was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), 600 µl was transferred to a fresh tube, 2 volumes of 100% ethanol was added, and the DNA was precipitated by microcentrifugation for 15 minutes at 4°C. The pellet was resuspended in 50 µl of TE (10 mM Tris pH 8.0, 1 mM EDTA) containing 100 µg/ml of RNAase A. Ten µl of DNA (approximately 100 ng) was digested in 100 µl total volume with appropriate enzymes, precipitated with 200 µl ethanol, and resuspended in 10 µl of DNA loading dye. The DNA was separated in 0.7% agarose gels and transferred to nylon membranes (Nytran N^+, Amersham Corp., Arlington Heights, IL) in a semi-dry blotting apparatus (BioRad Laboratories, Richmond, CA) as recommended by the manufacturer. Transferred DNA was denatured, neutralized, and cross-linked to the membrane with UV light using a Stratalinker (Stratagene, La Jolla, CA). To identify strains with a tandem integration at *PEP4*, two probes were used. One was a 1400 bp EcoRI - HindIII fragment from the 3' end of *PEP4*. The second was a 2000 bp BamHI - EcoRI fragment from the 5' end of *PEP4*. Fragments were detected using chemiluminescence reagents (ECL™ direct labelling kit; Amersham Corp., Arlington Heights, IL).

Parent strains harboring a tandem duplication of the wild-type and deletion alleles of the gene were grown in YEPD broth overnight to allow for the generation of looped-out, Ade^- strains. These cells were then plated at a density of 2000-5000 colonies per plate on adenine-limited YEPD plates, grown for 3 days at

30°C and 3 days at room temperature. The shift to room temperature enhanced pigmentation of rare, pink, Ade⁻ colonies. Loop-out strains were consistently detected at a frequency of approximately one pink, Ade⁻ colony per 10,000 colonies screened. These strains were screened for retention of the wild-type or mutant genes by Southern blotting or by PCR using primers that spanned the site of the deletion. An *ade2-11 pep4Δ* strain was designated PMAD15.

The *PRB1* gene was then deleted from PMAD15 essentially as described above by transformation with plasmid pCZR153. Blots were probed with PCR-generated probes for internal portions of the *PRB1* and *ADE2* genes. The *PRB1* probe was generated by subcloning a 2.6 kb ClaI - SpeI fragment of *PRB1* into the phagemid vector pBluescript® II KS(+) to produce pCZR150, and amplifying the desired region by PCR using primers ZC447 (SEQ ID NO:17) and ZC976 (SEQ ID NO:18). The ADE2 probe was generated by amplifying the ADE2 gene in pCZR139 with primers ZC9079 (SEQ ID NO:19) and ZC9080 (SEQ ID NO:20). The resulting *ade2-11 pep4Δ prb1Δ* strain was designated PMAD16.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

What is claimed is:

1. An isolated DNA molecule of up to 1500 nucleotides in length comprising nucleotide 810 to nucleotide 1724 of SEQ ID NO:1.

2. A DNA construct comprising the following operably linked elements:

a first DNA segment comprising at least a portion of the sequence of SEQ ID NO:1 from nucleotide 733 to nucleotide 1732, wherein said portion is a functional transcription promoter;

a second DNA segment encoding a protein of interest other than a *Pichia methanolica* glyceraldehyde-3-phosphate dehydrogenase; and

a third DNA segment comprising a transcription terminator.

3. The DNA construct of claim 2 wherein said first DNA segment is from 900 to 1500 nucleotides in length.

4. The DNA construct of claim 2 wherein the first DNA segment comprises nucleotide 810 to nucleotide 1724 of SEQ ID NO:1.

5. The DNA construct of claim 2 wherein the first DNA segment is essentially free of DNA encoding a *Pichia methanolica* glyceraldehyde-3-phosphate dehydrogenase.

6. The DNA construct of claim 2, further comprising a selectable marker.

7. The DNA construct of claim 2, further comprising a secretory signal sequence operably linked to the first and second DNA segments.

8. The DNA construct of claim 7, wherein the secretory signal sequence is a *Saccharomyces cerevisiae* alpha-factor pre-pro sequence.

9. The DNA construct of claim 2 wherein said third DNA segment comprises a transcription terminator of a *Pichia methanolica AUG1* or *GAP1* gene.

10. The DNA construct of claim 9, wherein said terminator comprises nucleotides 2735 to 2795 of SEQ ID NO:1.

11. A *Pichia methanolica* cell containing the DNA construct of claim 2.

12. The *Pichia methanolica* cell of claim 11 wherein the DNA construct is genomically integrated.

13. The *Pichia methanolica* cell of claim 12 wherein the DNA construct is genomically integrated in multiple copies.

14. The *Pichia methanolica* cell of claim 11 wherein the first DNA segment is from 900 to 1500 nucleotides in length.

15. The *Pichia methanolica* cell of claim 11 wherein the first DNA segment comprises nucleotide 810 to nucleotide 1724 of SEQ ID NO:1.

16. The *Pichia methanolica* cell of claim 11, wherein the cell is functionally deficient in vacuolar proteases proteinase A and proteinase B.

17. A method of producing a protein of interest comprising:

culturing the cell of claim 11 whereby the second DNA segment is expressed and the protein of interest is produced; and

recovering the protein of interest.

18. The method of claim 17 wherein the DNA construct is genomically integrated in multiple copies.

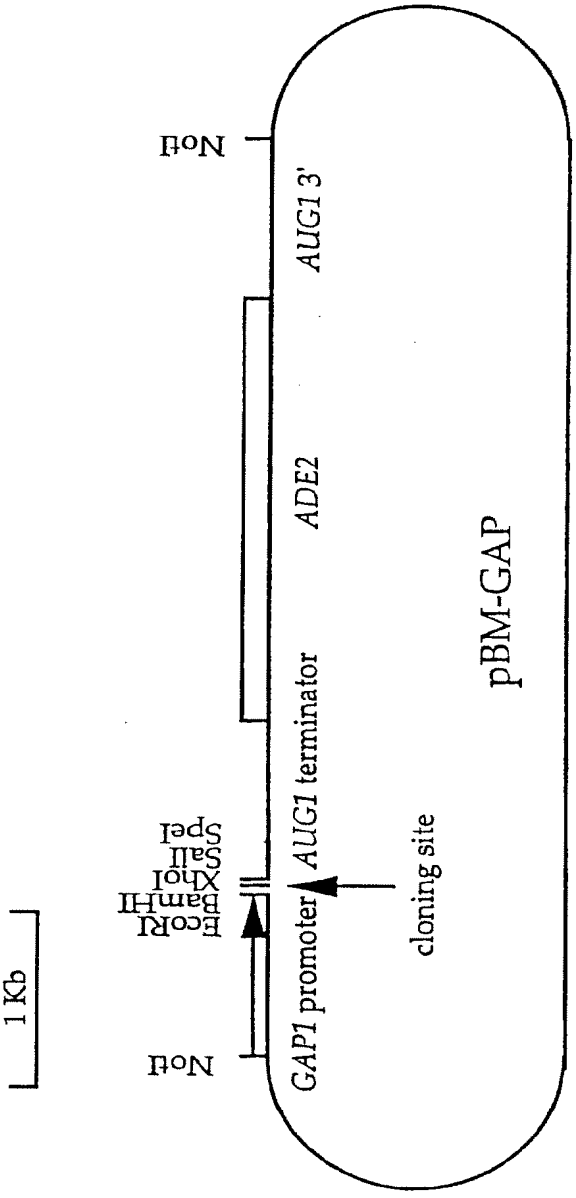
19. The method of claim 17, wherein the cell is deficient in vacuolar proteases proteinase A and proteinase B.

20. A DNA construct comprising the following operably linked elements:

a first DNA segment comprising a *Pichia methanolica* gene transcription promoter;

a second DNA segment encoding a protein of interest other than a *Pichia methanolica* protein; and

a third DNA segment comprising nucleotides 2735 to 2795 of SEQ ID NO:1.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ADE2
pBM-GAP
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SpeI
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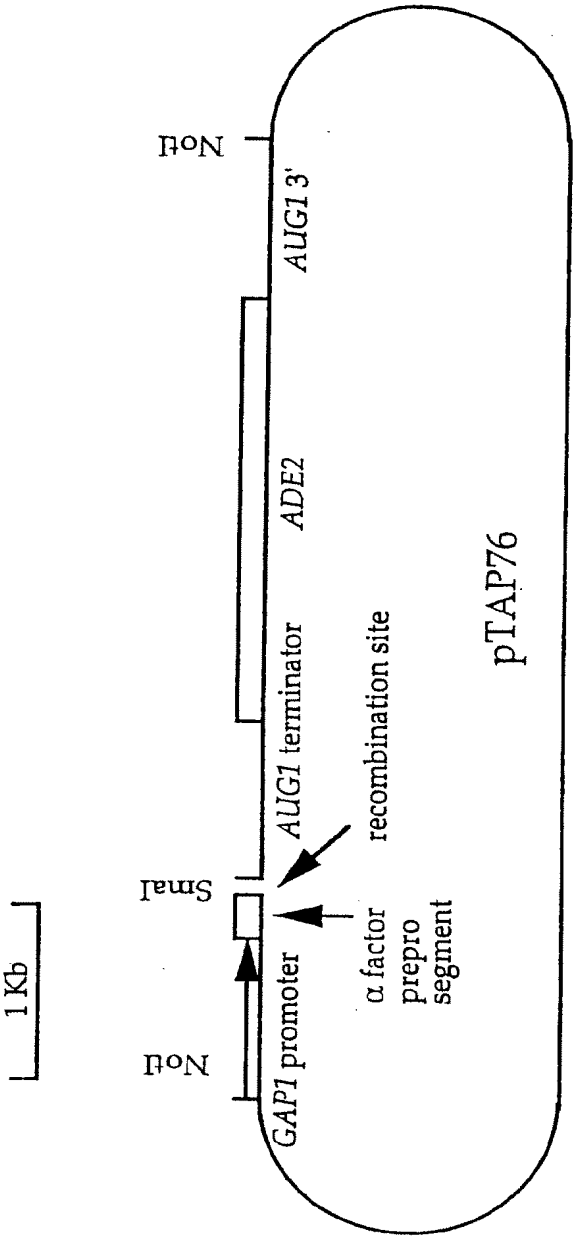
GAP1 promoter
NotI
1 Kb

Fig. 1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α factor
prepro
segment
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NotI
GAP1 promoter

Fig. 2

SEQUENCE LISTING

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Raymond, Christopher K.
Vanaja, Erica
Miller, Brady G.
Sloan, James S.

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Pro Lys Gln Asp Phe Ala Glu Ala Thr Ser Glu Pro Gly Leu Ala Phe
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Gly Val Glu Tyr Ala Pro Asn Ala His Leu Ala Glu Ala Lys Ala Asn
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                85                  90                  95

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 00/16671

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/81 C12N9/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 14347 A (ZYMOGENETICS INC) 25 March 1999 (1999-03-25) cited in the application	1-7,9-20
Y	page 13, line 13 - line 36	8,16
Y	WO 98 20035 A (UNIV AUTONOMA DE NUEVO LEON ;VIADER SALVADO JOSE MARIA (MX); BARRE) 14 May 1998 (1998-05-14) cited in the application abstract page 4, line 25 - line 31; claim 1	8
X	WO 97 17450 A (ZYMOGENETICS INC) 15 May 1997 (1997-05-15)	1-7, 9-15, 17-20
Y	page 12, line 18 -page 13, line 12 page 30, line 7 page 36, line 11 - line 12	8,16
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 October 2000	18/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

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INTERNATIONAL SEARCH REPORT

Int[illegible]onal Application No
PCT/US 00/16671

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 374 913 A (PHILLIPS PETROLEUM CO) 27 June 1990 (1990-06-27) the whole document	
A	EP 0 438 200 A (CIGB) 24 July 1991 (1991-07-24) abstract	
A	WATERHAM H R ET AL: "Isolation of the Pichia pastoris glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES,GB,ELSEVIER SCIENCE PUBLISHERS, BARKING, vol. 186, no. 1, 20 February 1997 (1997-02-20), pages 37-44, XP004054877 ISSN: 0378-1119 the whole document	
A	DATABASE GENBANK 'Online! GI=2995611, Acc.no. U95625, 28 March 1998 (1998-03-28) SOHN, J.-H. ET AL.: "Pichia angusta glyceraldehyde 3-phosphate dehydrogenase gene, complete cds." XP002149526 the whole document	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l Application No
PCT/US 00/16671

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			CU	22288 B	06-09-1994
			JP	5184352 A	27-07-1993